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1. Chien, J. et al. Mol. and Cell. Endocrinology (2001) 181(1-2): 69-79
2. Chien, J. et al. Int. J. of Cancer (2001) 91(1): 46-54
3. Chien, J. et al. Oncogene (1999) 18(22): 3376-3382
4. Wong, E.C.C. et al. Proc. Amer. Assoc. for Cancer Res. (1997) 38: 288
5. Rayford, W. et al. Prostate (1997) 30(3): 160-166
6. Xue-Zhang, Q. et al. Endocrine (1995) 3(6): 445-451
7. Shah, G.V. et al. Endocrinology (1994) 134(2): 596-602
8. Rayford, W. et al. J. of Urology (1994) 151(5 suppl): 490A
9. Rayford, W.et al. J. of Urology (1993) 149(4 suppl): 479A
10. Shah, G.V. et al. Prostate (N.Y.) (1992) 21(2): 87-97
11. Sagol, O. et al. Annals of Medical Sciences (1999) 8(1): 14-21
12. Sussenot, O. et al. Prostate (1998) 36(suppl. 8): 43-51
13. Hanna, F.W. et al. J. Endocrinol. (1997) 152(2): 275-281
14. Sim, S.J. et al. Annals of Clinical and Laboratory Science (1996) 26(6): 487-495
15. Watanabe, K. et al. Fukushim J. Medical Science (1995) 41(2): 141-152
16. Esik, O. et al. European J. Gynaecological Oncology (1994) 15(3): 211-216

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Tumor Heterogeneity and Neuroendocrine Differentiation in Non- Small Cell Lung Carcinomas; Investigation of Their Relation With Tumor Stage, P53 Protein and Pcna Expression

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Purpose: The biologic significance of tumour heterogeneity including neuroendocrine differentiation in non small cell lung carcinomas is not certain. The purpose of this study is to investigate whether there is any significant difference between non small cell carcinomas with and without tumour heterogeneity including neuroendocrine differentiation in terms of tumour stage, tumour suppressor gene alterations shown by p53 protein expression and proliferative activity shown by PCNA expression.

Methods: Paraffin sections of 57 non small cell carcinomas were reviewed and microscopic tumour heterogeneity was evaluated. The sections were all stained with neuroendocrine markers including Neuron Specific Enolase, Chromogranin A, Calcitonin and Serotonin antibodies for the evaluation of neuroendocrine differentiation, with anti-PCNA antibody for the evaluation of the proliferative activity of tumours, and with p53 antibody for the detection of mutant nuclear p53 protein expression. Standard streptavidin biotin immunperoxidase method was used for immunohistochemical staining. Fifty seven cases were graded for cytoplasmic staining with NE markers on a scale of 0 (-), to 3 positive(+). Nuclear staining for p53 was semiquantitatively graded as follows: (-) : no staining, 1+: 1-30% of cells stained, 2+ : 30-70% of cells stained, 3+: 70% or more stained cells. Nuclear staining for PCNA was evaluated by counting 1000 tumour cells and graded as follows:1+: 1-40% of cells stained, 2+: 40-75 % of cells stained, 3+:75 % or more cells stained.

Results: Tumour heterogeneity was found in 12 % of non small cell lung carcinomas on routine sections. Tumours with and without heterogenous areas did not show statistically significant difference in terms of tumour

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stage, proliferative activity and p53 expression. five percent of 57 non-small cell carcinomas reacted with two neuroendocrine markers. 42 % of cases were positive for only one neuroendocrine marker. There was no statistically significant difference between the cases which were negative and cases which were positive with 1 or 2 neuroendocrine markers, in terms of proliferative activity and p53 expression .

Seventy -one percent of 57 non small cell carcinomas reacted with p53 antibody. Cases with p53 protein expression were not different from negative cases in terms of proliferative activity, neuroendocrine differentiation and tumour stage. All cases of non small cell carcinomas reacted with PCNA antibody with the mean staining index of 61.6 %. PCNA staining indices did not show statistically significant difference between subtypes and tumour stages.

Conclusion: A complete NE differention demonstrated by positivity for multiple NE markers similar to NE tumours is quite unusual. Heterogeneity and neuroendocrine differentiation in NSCLCs shown by light microscopy or by one or two NE marker positivity does not correlate with tumours stage which is the most valuable predictor of prognosis and with other factors like p53 and PCNA reactivity whose influence on prognosis is debatable. Ann *Med Sci 1999;8:14-21*

Key words: *Non small cell lung cancer, tumour heterogeneity, neuroendocrine differentiation, p53, PCNA.*

Microscopic tumour heterogeneity defined as the presence of different histologic types within the same tumour is reported in 13-63% of lung cancers.[1,2,3] As a practical manner most lung cancers can be classified as either I- non-small cell lung cancers (NSCLC) including squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma and large cell carcinoma or II- small cell lung cancers (SCLC).[1,4,5] While the presence of small cell carcinoma component in NSCLC, regardless of the extent of it, implies poor prognosis and different treatment, the clinical significance of coexistence of different types of NSCLCs is unclear.[6]

Neuroendocrine (NE) differentiation detected by light microscopy or immunhistochemistry has been reported in 10-50 % of NSCLCs.[4,5,7,8] Neuroendocrine differentiation is most commonly reported in large cell carcinomas and poorly differentiated adenocarcinomas.[5,7,9,10] Recent studies have shown conflicting results regarding both the criteria for definition and the clinical significance of NE differentiation.[5,9]

Current advances in molecular biology revealed that mutations of the P53 gene are one of the most requent genetic abnormalities in human neoplasms including lung cancer.[11,12] P53 gene mutations were reported in 37-80 % of all types of lung cancer.[13,16] In some studies mutation of p53 gene has been reported to be associated with poor prognosis, while in others conflicting results have been reported.[5,13,17-25] There are also confusing results on the relation of mutant p53 protein expression with the stage of the disease.[5,26,27] Besides, it has been suggested in some reports that the mutant p53 gene enhances proliferative activity by inducing PCNA gene and that the p53 protein expression increases with the degree of neuroendocrine differentiation in NE carcinomas.[13,28]

Proliferative activity expressed by PCNA expression was reported to be high in lung carcinomas.[29] The prognostic value of PCNA expression in lung cancer is not clear.[30-32]

The aim of this study is to investigate the microscopic heterogeneity and NE differentiation in NSCLC as it was shown by immunohistochemical demonstration of NE markers and to search for any difference in p53 tumour suppressor gene mutation, proliferative activity and tumour stage between NE marker positive and negative tumors. Besides, the relation of p53 expression and proliferative activity of tumours were evaluated.

Materials and Methods

Formalin fixed and paraffin embedded archival material of 57 lung carcinomas diagnosed in our department between the years 1991 and 1995 were included in this study. Pathologic material was obtained by endoscopic biopsy in 24 of cases, and by lobectomy in 33 cases. The cases were staged according to TNM system[33].

Eight to 10 Haematoxylin and Eosin (H&E) sections from each tumour excised by lobectomy

and endoscopic biopsy materials were screened to assess histologic subtype according to WHO classification.[34] Tumour heterogeneity defined as the presence of different histologic types within the same tumour was evaluated in each tumour. Five micron sections from selected representative paraffin blocks were taken on poly-l- lysine coated slides. Paraffin sections were stained with NE markers including Neuron Specific Enolase (NSE), (DakoH14, 1/100 dilution)) , Chromogranin A (Dako Kr A, Dak-A3, 1/100 dilution), Calcitonin (Dako,1/200 dilution), Serotonin (Dako- 5HT- H209,1/10 dilution) for the evaluation of NE differentiation. The sections were stained with anti-PCNA antibody (Dako,1/100 dilution) for the evaluation proliferative activity of tumours, and with p53 antibody (Dako, DO7,1/50 dilution) for detection of mutant nuclear p53 protein. The streptavidin biotin immunperoxidase method was used for immunohistochemical staining. A microwave antigen recovery technique requiring the boiling of the deparaffinised sections in 0.01 M citrate was used prior to immunostaining. Diaminobenzydine was used as a chromogen. Carcinoid tumour of the lung known to express all NE markers used in this study and a breast carcinoma section known to express p53 protein and PCNA antibodies were used as positive controls. Negative controls were provided by omitting of primary antibody during the staining procedure.

Fifty seven cases were graded for cytoplasmic staining with NE markers on a scale of 0 (-), to 3 positive(+). (-) indicated no staining, 1+, rare scattered individual cells with positively stained cytoplasm, 2+, small clusters of stained cells and 3+, extensive staining. Nuclear staining for p53 was semiquantitatively graded as follows: (-) : no staining, 1+:1-30% of cells stained, 2+ : 30-70% of cells stained, 3+: 70% or more stained cells.

Nuclear staining for PCNA was evaluated by counting 1000 tumour cells and graded as follows: 1+: 1-40% of cells stained, 2+: 40-75 % of cells stained, 3+: 75 % or more cells stained.

The results of this study were evaluated statistically by using Rank correlation, Kruskal Wallis, Kolmogorov Smirnof and Chi- Square tests.

Results

Ages of the 57 patients with NSCLC ranged from 34 to 77(mean: 55.5) years. Six of the patients were females and 51 were males. According to the TNM staging system, 18 cases were in stage 1, 6 cases were in stage 2, 7 were in stage 3 A, 10 were in stage 3B and 16 cases were in stage 4. According to the WHO classification[34], 38 of cases were squamous cell carcinomas[22] well differentiated, 4 moderately differentiated and 12 poorly differentiated), 14 were adenocarcinomas (10 well differentiated, 2 moderately differentiated and 2 poorly differentiated), 2 were adenosquamous carcinomas and 3 were large cell carcinomas. 7 (12.28%) of 57 NSCLC cases showed tumour heterogeneity. Two of these cases were adenosquamous carcinomas and 5 were squamous cell carcinomas with focal adenoid structures. Tumour heterogeneity was found in only resection materials (19.4%). Table 1 shows the distribution of tumour stage, p53 expression and PCNA positivity in heterogenous and nonheterogenous NSCLCs. Table 2 shows staining with NE markers, p53 and PCNA antibodies and stages of the cases.

Table 1. p53 and PCNA expression in heterogenous and non heterogenous NSCLCs

	Stage					PCNA score*			P53 score**			
	1	2	3 a	3b	4	1	2	3	0	1	2	3
Heterogenous (n:7)	4	1	1	1	0	1	3	3	2	2	0	3
Pure (n:50)	14	4	7	9	16	13	13	22	14	5	10	19

* Two cases were insufficient for PCNA staining.
** Two cases were insufficient for P53 staining.

Table 2. Stages, p53 and PCNA scores and NE marker positivity in NSCLCs

Number of positiveNE markers	Stage					P53scores*				PCNA scores**			Total
	1	2	3a	3b	4	0	1	2	3	1	2	3	
0	11	3	5	4	7	8	5	7	10	7	9	14	30
1	7	2	2	5	8	7	1	3	11	6	6	10	24
2	0	1	0	1	1	1	1	0	1	1	1	1	3

* 2 cases are insufficient for P53 staining.
** 2 cases are insufficient for PCNA staining.

Table 3. PCNA staining indices for NSCLCs

N:55	PCNA staining indices (%of positive stained nuclei)
Minimum	3.2
Maximum	99.2
mean	61.65
Median	64.00
Standard deviation	23.84

Thirty (52.63%). of 57 NSCLC s showed no staining for any of the NE markers. Twenty - four (42.1%) cases were stained for only 1 NE marker. (2 of the cases were stained with only calcitonin, 22 were stained with only NSE) (28%=+,56%=2+,16%=3+). Two squamous cell carcinomas and 1 adenosquamous carcinomas (5.26%) showed staining with both NSE and calcitonin. None of our cases were stained with antibodies against serotonin or chromogranin. Thirty-nine (70.91) % of 57 NSCLCs were stained with P53 antibody. Two of the cases were found to be insufficient for evaluation. Fifty-five of 57 (96%) cases were stained positive with PCNA antibody. Again, 2 cases were found to be insufficient for evaluation. PCNA staining indices are shown in table 3.

As a result no statistically significant difference was found between the heterogenous and the non heterogenous tumours in terms of tumour stage, mutant p53 protein expression and proliferative activity. There was also no statistically significant difference between tumours with no NE marker positivity and tumours stained with 2 NE markers or with 1 marker only, in terms of tumour stage, p53 expression and proliferative activity. P53 positive and negative cases showed no difference in terms of tumour stage. No relation was found between the scores of staining and stage of tumour in p53 staining. There were also no relation between PCNA indices, tumour stage and histologic type as well as between tumours with high PCNA indices and tumours with low indices in terms of p53 scores.

Discussion

The histopathologic heterogeneity has been reported within an individual tumour in up to 10-63 % of sufficiently sampled lung cancers by light microscopy alone. In some studies, non-small cell lung cancer heterogeneity was found to correlate with tumour stage[1,2,3]. In this study, microscopic heterogeneity was detected in 12.8% of NSCLCs and heterogenous tumours did not show statistically significant difference in terms of tumour stage, proliferative activity, p53 mutations and NE differentiation suggesting that the dominant pattern determines the tumor behaviour in NSCLCs. Our finding of heterogeneity by light microscopy in only resection materials, demonstrates the need for sufficient material in order to recognize tumour heterogeneity.

In addition, up to half of NSCLCs which by light microscopy have typical features of adenocarcinoma, squamous cell carcinoma or large cell carcinoma and lack light microscopic features of NE differentiation may demonstrate focal or more extensive positivity for a NE marker[4,5,7-10,35]. NE differentiation was found in 3- 50% of NSCLCs[5,7,8,36]. NE differentiation are most commonly reported in large cell and squamous cell carcinomas[5,7,9,10]. NSE, Chromogranin, Serotonin, Calcitonin, Synaphtophysin and Leu7 are the most commonly used immunohistochemical NE markers to reveal neuroendocrine features. Among the NE markers used in this study, NSE is an isoenzyme of a glycolytic enzyme enolase which is found as dimers of alpha, beta and gamma subunits. Gamma- gamma dimers are thought to be spesific for neural tissues although in some recent reports it's claimed that this dimer can be found in non neural tissues[37]. Chromogranin is an acidic protein found in cytoplasmic granules. The role of chromogranins is uncertain although there is some evidence that they take part in the maturation of granules, regulation of enzyme activity of granules and peptide hormone synthesis[38-39]. Calcitonin and serotonin are specific products secreted by NE cells[7]. Whether positivity with only one of these markers represents NE differentiation or not, is debatable.

In many studies, NSE positivity alone in NSCLCs was reported to be nonspecific and insufficient for indicating NE differentiation. In some studies positivity with only one marker other than NSE was thought to be sufficient to indicate NE differentiation.[40] Linnolia et al used 9 NE markers in their study and regarded the tumours which were positive for 4 or more NE markers as tumours with NE differentiation. In the latter study it is believed that tumours which are indistinguishable from NE tumours immunohistochemically should be called tumours with NE differentiation[7]. In the study of Brambilla et al, 3 NE marker positivity was accepted to be sufficient to indicate NE differentiation[5]. On the other hand, there are studies accepting 2 or more marker positivity[4,9]. In an electron microscopic (EM) study of Wilson et al, it was shown that all of the tumours with dense core granules detected by EM, showed immunohistochemically positive reaction with NSE, while others without dense core granules failed to react, indicating that NSE might indeed rep-

resent NE differentiation[8]. It has been suggested that NSE detects cells with minimal NE differentiation.(ie. those with few secretory granules or low levels of hormones) that can not be detected by other techniques[41].

In any case, the clinical significance of NE differentiation either shown by NSE positivity alone or a combination of markers is a subject of controversy. In some studies, it has been claimed that, NSE immunoreactivity correlated with improved surviva[9]. Graziano et al and Scov et al reported that NSCLCs with NSE positivity showed increased response to chemotherapy[8]. However in some other studies, no such relation had been found between NE differentiation and tumours stage, relapse and survival in NSCLCs.

Figure 1. Cytoplasmic staining with calcitonin in adenosquamous carcinoma (X100)

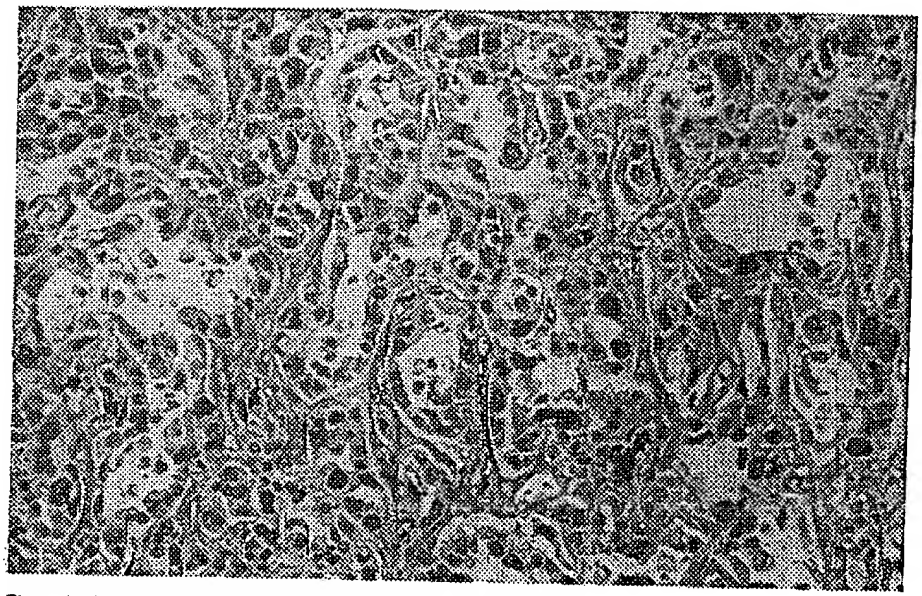

Figure 2. Nuclear staining with P53 antibody in adenocarcinoma (X100)

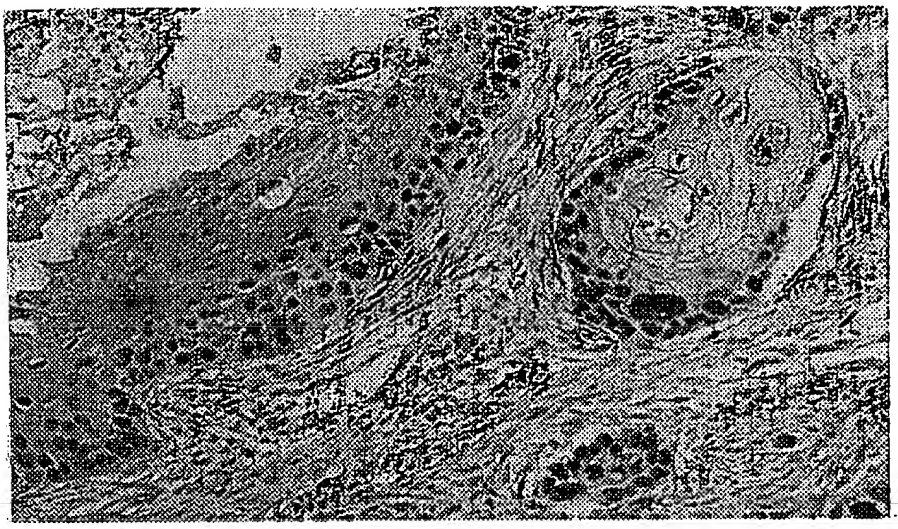

Figure 3. Nuclear staining with PCNA antibody in a squamous cell carcinoma. (X100)

In the present study, there are only three cases immunoreactive for more than one marker. On the other hand the finding of NSE positivity in 43,8% (25 of 57 cases) of cases is in accordance with previous reports. We have found no statistically significant difference between stages of NSCLCs with and without NSE immunreactivity in the tumour tissues .

The heterogeneity of lung cancers, including NE differentiation in NSCLCs is currently being explained by an hypothesis that all lung cancers are ultimately derived from a common stem cell which in addition to undergoing malignant transformation, can potentially differentiate into any of the different cell types or into combined types[3]. It's also believed that the phenotype of the lung cancer, including not only the cell types but other characteristics such as growth rate, metastatic potential and response to therapy represents the expression of the specific genetic abnormalities. Abnormalities of tumour suppressor gene P53, ranging from complete deletion to point mutation, constitute some of the most frequently encountered genetic defects in human cancer including lung cancer[20,23].

P53 is a tumour suppressor gene encoding 53 kD intranuclear protein which is known to regulate transcription, DNA replication and induces apoptosis. This protein can not be detected by immunohistochemistry in normal conditions but mutations of p53 gene stabilises the p53 protein and makes it accumulate in the nucleus and be detected by immunohistochemistry[22,42-44]. Mutations in p53 gene is detected in 37 to 80% of all lung carcinomas and there seems to be no predilection of p53 mutation for any specific phenotype [13,16,19,26,45]. In the present study, 70.91% of NSCLC cases reacted with p53 antibody immunohistochemically. Unlike some studies suggesting that mutations appear in early stages of the disease, p53 positivity and the degree of the staining did not vary between different stages indicating that mutations can appear in every stage of the disease[5,46,47].

There are only a few studies investigating the relation between p53 mutation and NE differentiation. Barbareski et al. reported that with decreasing NE differentiation from carcinoid to small cell carcinoma, the frequency of p53 mutation and PCNA activity is increased[13,28]. On the basis of such observations, it can be speculated that p53 mutation might be responsible for loss of NE features while contributing malignant transformation. If that is the case, than p53 mutation in NSCLC s with NE differentiation could be expected to be less frequent than the ones without NE differentiation . In order to investigate the validity of this suggestion, we compared the frequency of p53 mutations and proliferative activity in NE marker positive and negative NSCLCs. Our results did not show any statistically significant difference in p53 mutation or proliferative activity between the NE marker positive (one or two marker positivity) and negative cases. One reason for these findings might be minimal NE differentiation in our cases, most of which had had only NSE positivity . It might be more meaningful to compare the p53 expression of NSCLCs or SCLCs with varying degrees of NE differentiation to test the validity of this hypothesis.

Proliferative indices in our cases are consistent with the study of Theunissen at al, indicating that the proliferative activity is high in lung carcinomas[29]. In our study no statistically significant relation could be found between the proliferative indices and tumour stage and histologic subtypes. Lee at al also found no relation

between PCNA indices and tumour stage while Ishida et al found statistically significant relation[48]. Haerslev et al, found that, 52% of the lung tumours included in their study had high proliferative activity and like Ebina et al, they found positive correlation between p53 mutations and PCNA positivity[16,18]. Korkolopoulau et al also found positive correlation between p53 expression and PCNA indices[13]. They indicate that wild type p53 gene selectively suppresses the PCNA m RNA and PCNA protein in the nucleus and that, mutant p53 gene has an inducing role on p53 gene[13]. Fontanini et al, did not find any relation between p53 gene and PCNA expression[49]. In our study in consistence with the latter study no correlation has been detected between PCNA indices and p53 protein expression .

In conclusion, although NSE positivity which may reflect minimal NE differentiation, is not unusual in NSCLCs. It appears that a complete NE differentiation demonstrated by positivity for multiple NE markers similar to NE tumours is quite unusual. The genetic basis for such a differentiation remains to be investigated. Heterogeneity and neuroendocrine differentiation in NSCLCs shown by light microscopy or by one or two NE marker positivity does not correlate with tumours stage which is the most valuable predictor of prognosis and with other factors like p53 and PCNA reactivity whose influence on prognosis is debatable.

References

1. Carter, N., Nelson, F., Gosney, J. Ultrastructural heterogeneity in undifferentiated bronchial carcinoma. Journal Of Pathology, 1993;171: 53-57.
2. Fraire, A.E., Roggli, V.L., Volmer, R.T., Greenberg,S.D., McGavran, M.H., Spjut,H.J., Yesner, R. Lung cancer heterogeneity. Cancer, 1987, 1(60): 373-375.
3. Mc Dowell, E.M., Beals, T.F eds. Biopsy pathology of the bronchi. Philadelphia: Saunders;.1987;308-367
4. ASCP. Check Sample ; AP - 90-3 ; 2-5.
5. Brambilla, E., Gazzeri, S., Moro, D., Fromentel, C., et al. Immunohistochemical study of p53 in human lung carcinomas. Am J Pathol, 1993;143:199-210.
6. Radice, P.A., Matthews, M.J., Ihed, D.C., Gazdar , A.F., et al. The clinical behavior of " Mixed " small cell/ large cell bronchogenic carcinoma compared to" pure" small cell subtypes. Cancer ,1982;50:2894-2902.
7. Linnolia , R.L., Mulshine , J.L., Steinberg.,M., Funa, K., et al. Neuroendocrine differentiation in endocrine and nonendocrine lung carcinomas. Am J Clin Pathol ,1988; 90:641-652.
8. Wilson, T.S., McDowell, E.M., VetMed, B., Marangos, P.J., et al. Histochemical studies of dense-core granulated tumours of the lung. Arch Pathol Lab Med ,1985;109:613-620.
9. Graziano, S.L., Tatum, A.H., Newman, N.B., Oler , A., et al. The prognostic significance of neuroendocrine markers and carcinoembryonic antigen in patients with resected stage I and II non-small cell lung cancer. Cancer Research,1994;54:2908-2913.
10. Visscher, D.W., Zarbo, R.J., Trojanowski , J.Q., Sakr, W., Crissman, J.D. Neuroendocrine differentiation in poorly differentiated lung carcinomas: A light microscopic and immunohistologic study. Modern Pathology, 1990;3(4):508-512.
11. Iggo, R., Gatter, K., Bartek, J., Lane, D., Harris, A.L. Increased expression of mutant forms of p 53 oncogene in primary lung cancer. Lancet, 1990; 335:675-679.
12. Roth, J.A. Modulation of oncogene and tumour-suppressor gene expression: A novel strategy for cancer prevention and treatment. Ann surg Oncol , 1(1); 79-86.
13. Korkolopoulou , P., Oates , J., Crocker , J., Edwards, C.. P 53 expression in oat and non-oat small cell lung carcinoma:correlations with proliferating cell nuclear antigen. J Clin Pathol , 1993;46:1093-1096.
14. Lee, L.N., Shew ,J.Y., Sheu, J.C., Lee, Y.C., Lee, W.C., et al. Exon 8 mutation of p53 gene associated with nodal metastasis in non-small-cell lung cancer. Am J Respir Crit Care Med.,1994;150(6):1667-1671.
15. Marchetti, A., Buttitta , F., Merlo, G., Diella, F., et al. P53 alterations in non-small cell lung cancer correlate with metastatic involvement of hilar and mediastinal lymph nodes. Cancer Res., 1993;53(12):2846-2851.
16. Takahashi, T., Carbone, D., Takahashi, T., Nau, M.M. , et al. Wild-type but not mutant p 53 supresses the growth of human lung cancer cells bearing multiple genetic lesions. Cancer Resarch , 1992;52:2340-2343.
17. Casson , A.G., McCuaig , S., Craig, I., Ayed , A., et al. Prognostic value and clinicopathologic correlation of p53 gene mutations and nuclear DNA content in human lung cancer. J Surg Oncol,.1994;56(1):13-20.
18. Haerslev, T., Jacopsen, G.K. An immunhistochemical study of p53 with correlations to histopathological parameters , c-erb-B2 , proliferating cell nuclear antigen and prognosis. Hum Pathol, 1995;26:295- 301.
19. Harpole , D.H., Marks, J.R., Richards, W.G., Herndon, J.E., Sugarbaker, D.J. Localized adenocarcinoma of the lung: Oncogene expression of c-erbB-2 and p53 in 150 patients. Clinical Cancer Research., 1995; 1; 659-664.
20. Horio , Y., Takahashi, T., Kuroishi, T., Hibi, K., et al. Prognostic significance of p53 mutations and 3p deletions in primary resected non small cell lung cancer. Cancer Research, 1993;53:1-3.
21. Johnson , B., Kelley, M.J. Overview of genetic and molecular events in the pathogenesis of lung cancer. Chest, 1993;103: 1S -3S.
22. Mac Laren, R., Kuzu, I., Dunnill , M., Harris, A., et al. The relationship of p53 immunostain-

ing to survival in carcinoma of the lung. Br J Cancer , 1992; 66: 735- 738.
23. Minna, J. The molecular biology of lung cancer pathogenesis. Chest, 1993; 103: 449 S-456 S
24. Mitsudomi,T., Oyama,T., Kusano,T., Osaki,T., et al. Mutations of the p53 gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. J Natl Cancer Inst.,1993;(24):2018-2023.
25. Quinlan , D.C., Davidson, A.G., Summers , C.L., Warden, H.E., et al. Accumulation of p53 protein correlates with a poorer prognosis in human lung cancer. Cancer Research , 1992; 52: 4828-4831.
26. Winter, S.F,. Minna, J.D.,Jhonson ,B.E., Takahashi, T., Gazdar, A.F., Carbone, D.P. Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. Cancer Research, 1992;52:4168-4174.
27. Kishimoto , Y., Murakami , Y., Shiraishi , M., Hayashi , K. , et al. Aberrations of the p53 tumor suppressor gene in human non small cell carcinomas of the lung. Cancer Research, 1992; 52: 4799- 4804.
28. Barbareschi , M., Girlando, S., Mauri, F.A., Arrigoni, G., et al. Tumor supressor gene products, proliferation and differantiation markers in lung neuroendocrine neoplasms. Journal of Pathology , 1992;166:343-350.
29. Theunissen, P.H.M.H., Leers , M.P.G., Bollen, E.C.M. Proliferating cell nuclear antigen (PCNA) ekspression in formalin -fixed tissue of non small cell lung carcinoma. Histopathology,1992; 20: 251- 255.
30. Ebina, M., Steinberg, S., Mulshine, J.L., Linnolia, R.I. Relationship of p53 overexpression and up regulation of proliferating cell nuclear antigen with the clinical course of non small cell lung cancer. Cancer Research 1994; 54:2496-2503.
31. Ishida , T., Kaneko, S., Akazava , K., Tateishi, M ., et al. Proliferating cell nuclear antigen ekspression and argyrophilic nucleolar organizer regions as factors influencing prognosis of surgically treated lung cancer patients. Cancer Research ,1993;53: 5000-5003..
32. Ogawa, J., Tsurumi , T., Yamada, S., Koide, S., et al. Blood vessel invasion and expression of Sialyl Lewis and proliferating cell nuclear antigen in stage I non small cell lung cancer. Cancer , 1994;73:1177-83.
33. Myers JL. Askin FB Youshem SA, Recommendations for the reporting of resected primary lung carcinomas. Hum Pathol ,1995; 26 (9):937-939.
34. American Society of Clinical Pathologists. The world health organisation histological typing of lung tumours. Am J Clin Pathol., 1982; 77:123-136.
35. Mardini, G., Pai, U., Chavez, A.M., Tomashefski, J.F. Endobronchial adenocarcinoma with endometrioid features and prominent neuroendocrine differentiation. Cancer, 1994;73:1383-1389.
36. Guinee , D.G., Fishback, N.F., Koss, M.N., Abbondanzo, S.L., Travis, W.D.. The spectrum of immunohistochemical staining of small-cell lung carcinoma in specimens from transbronchial and open-lung biopsies. Am J Clin Pathol \1994;102:406-414.
37. Perentes, E., Rubinstein, L.J. Recent applications of immunoperoxidase histochemistry in human neurooncology An update . Arch Pathol Lab Med , 1987; 111: 796-812.
38. Kameya, Toru., Yamaguchi ,K.. The endocrine lung. In: Kovaks, K., Asa ,SL. eds. Functional Endocrine Pathology, USA : Blacwell scientific publications ; 1991:478-491.
39. Tötsch , M,. Müller, L.C., Hittmair , A., Öfner, D., Gibbs, A.L., et al. Immunohistochemical demonstration of chromogranin A and B in neuroendocrine tumors of the lung. Hum Pathol, 1992;23:312-316.
40. Schleusener, J.T., Tazelaar, H.D., Jung , S., Cha, S. S., Cera, P. J., Myers, J. et al : "Neuroendocrine differentiation is an independent prognostic factor in chemotherapy - treated non-small cell lung carcinoma". Cancer ,1996; 77, 1284-1291 .
41. Di Santagnese, PA., De Mesy Jensen ,K.L. Neuroendocrine differentiation in prostatic carcinoma. Hum Pathol,1996; 18: 849-856.
42. Dosaka Akita , H., Shindoh , M., Fujino , M., Kinoshita, I., et al . Abnormal p 53 expression in human lung cancer is associated with histologic subtypes and patient smoking history. Am J Clin Pathol , 1994;102:660-664.
43. Fujino, M., Akita , H.D., Kato, M.., Kinoshita, I ., et al. Simultaneus use of the PCR-SSCP metod and immunohistochemistry of increasing the detection efficacy of p53 abnormalities in human lung cancer. Am J Clin Pathol, 1995; 104: 319 -324.
44. Kerr , J.F.R., Winreford, C.M., Harmon, B.V. Apoptosis. Cancer , 1994; 73:2013-2026.
45. Lohmann , D., Putz , B., Reich, U., Bohm , J., et al. Mutational spectrum of the p53 gene in human small-cell lung cancer and relationship to clinicopathological data. Am J Pathol, 1993;142(3):907-915.
46. Boers , J.E., Ten, Velde G.P.M., Thunnissen, F.B.J. P53 in squamous metaplasia : A marker for risk of respiratory tract carcinoma. Am J Respir Clin Care Med ,. 1996; 153:411-6.
47. Rusch, V., Klimstra, D., Linkov, I., Dmitrovsky , E. Aberrant expression of p53 or the epidermal growth factor receptor is frequent in early bronchial neoplasia, and co-expression preceeds squamous cell carcinoma development. Cancer research, 1995; 55: 1365 -1372.
48. Lee, K.J., Lee, M.S., Kim, J.O., Kim, S.Y.A comparative study on aberations of chromosome 17 and proliferating cell fraction in lung cancer. J Korean Med Sci.,1993;8(4):271-280.
49. Fontanini, G., Bigini, D., Vignati, S., et al. P53 expression in non small cell lung cancer clinical and biological correlations. Anticancer Res, 1993;13 (3):737-42.

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